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LOW-TEMPERATURE MAGNETIC CIRCULAR DICHROISM EVIDENCE FOR THE CONVERSION OF FOUR-IRON-SULPHUR CLUSTERS IN A FERREDOXIN FROM *CLOSTRIDIUM PASTEURIANUM* INTO THREE-IRON-SULPHUR CLUSTERS

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Oxidation of the 8Fe ferredoxin from *Clostridium pasteurianum* with potassium ferricyanide, followed by purification on Sephadex G-25 and DE-23 cellulose columns, gives a protein with an intense EPR signal at g 2.01. The low-temperature magnetic circular dichroism (MCD) spectra of this species are different from those of the oxidized high-potential iron protein from *Chromatium* but identical with the spectra of ferredoxin II from *Desulphovibrio gigas*. On reduction of the ferricyanide-treated ferredoxin with sodium dithionite only a weak EPR signal with g factors of 2.05, 1.94 and 1.89 is obtained. The low-temperature MCD spectra are strongly temperature dependent with a form similar to those of dithionite-reduced *D. gigas* ferredoxin II. The MCD magnetization curves are dominated by a species with ground-state effective g factors of $g_{/\!/}$ 8.0 and $g_{\perp}$ 0.0, which are also similar to those determined recently by low-temperature MCD spectroscopy for *D. gigas* ferredoxin II. The MCD characteristics are quite different from those of dithionite-reduced ferredoxin from *Cl. pasteurianum*, untreated with ferricyanide. This establishes the close similarity of the iron-sulphur clusters in ferricyanide-treated *Cl. pasteurianum* ferredoxin and in *D. gigas* ferredoxin II. The latter is known to contain a single 3Fe centre, similar to that observed in ferredoxin I from *Azotobacter vinelandii* by X-ray crystallography. Therefore, it is concluded that the [4Fe-4S] clusters of *Cl. pasteurianum* ferredoxin are converted to 3Fe clusters on oxidation with ferricyanide.

Introduction

The presence of the [4Fe-4S] cluster in a range of proteins is now well established by a combination of X-ray crystallography [1,2] and spectroscopic techniques such as Mössbauer and EPR spectroscopy [3,4]. X-ray crystallography has revealed the presence of a single 4Fe cluster in the high-potential protein from *Chromatium vinosum* [1] and also the presence of two 4Fe clusters in the low-potential 8Fe ferredoxin from *Peptococcus aerogenes* [2]. The same structure is assumed to be present in the 8Fe ferredoxin from *Clostridium pasteurianum* [3]. This assumption is based upon the similarity of the EPR spectra of the clusters in this protein and the close homology of the amino acid sequence to those in *P. aerogenes* [3]. Both types of centre, namely, the high-potential and the low-potential cluster, undergo a one-electron redox cycle. When oxidized the high-potential protein from *C. vinosum* is paramagnetic and EPR detectable at temperatures below about 30 K with g factors of 2.12 and 2.04 and

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when reduced, diamagnetic at low temperatures [4]. By contrast, the [4Fe-4S] clusters of the ferredoxins of *P. aerogenes* and *Cl. pasteurianum* are diamagnetic, at low temperature, in the oxidized state and paramagnetic in the reduced state [3]. The paramagnetism is detectable by EPR spectroscopy with rather complex spectra being obtained, probably due to spin-spin coupling between the two clusters [5]. However, the average g value is about 1.94, below the spin-only value of 2.00, compared with values for oxidized high-potential protein which are above 2.00. These magnetic properties have been rationalized by the three-oxidation-state hypothesis of Carter et al. [6]. According to this scheme, the [4Fe-4S] cluster can adopt one of three possible oxidation levels differing by one electron per cluster. Hence, the oxidized high-potential proteins should contain a cluster isoelectronic with the $[Fe_4S_4(S\text{-}Cys)_4]^-$, where S-Cys is the anion of cysteine. This state, denoted C^{3+}, is paramagnetic with spin $S = 1/2$. One-electron reduction gives reduced high-potential protein, in which the cluster, now in oxidation state denoted C^{2+}, is diamagnetic at low temperature [3,7]. This one-electron reduction process takes place at a redox potential $E_0 \approx +350$ mV [8]. However, further one-electron reduction of the protein to produce an oxidation level C^+ is only possible if the protein is partially denatured in 80% dimethyl sulphoxide [9]. The clusters in the 8Fe ferredoxins, in contrast, undergo the one-electron reduction from states C^{2+} to C^+ at redox potentials of between -350 and -450 mV [10]. The C^+ state is EPR active with g values of approx. 2.06, 1.92 and 1.88. The oxidized C^{2+} state of 8Fe ferredoxins, and also 4Fe ferredoxins from anaerobic bacteria such as *Bacillus polymyxa* and *B. stearothermophilus*, is diamagnetic at low temperatures [3]. This is shown most clearly by the Mössbauer spectra [3]. At room temperature there is thermal population of paramagnetic states leading to magnetic moments of about 3.4 BM [11] and to contact-shifted resonances in the NMR spectrum of the $-CH_2-$ protons of the cysteine anion [11]. However, there is invariably a weak signal in the EPR spectrum below 35 K at g 2.01, which is almost isotropic [4]. This signal has been reported in a number of ferredoxins of the clostridial type [12]. The signal increases approx. 100-fold upon treatment of the ferredoxins with potassium ferricyanide, retaining the same g value and a similar line-width [12]. However, this signal is less easily power saturated than that from native ferredoxin [12]. It was concluded that the intense signal obtained after ferricyanide treatment was due to the [4Fe-4S] clusters in the C^{3+} or superoxidized oxidation level [12].

Inorganic models of oxidation levels C^{2+} and C^+ have been prepared and characterized by X-ray crystallography, Mössbauer and EPR spectroscopy, and magnetic susceptibility [13,14]. The compounds are of the general form $[R_4N]_2[Fe_4S_4(S\text{-}R)_4]$ and $[R_4N]_3[Fe_4S_4(S\text{-}R)_4]$, where R_4N is a tetralkyl ammonium cation and S-R is a range of organic thiol anions. The essential similarity of the spectroscopic properties of these clusters in solution to those of the protein-bound clusters leaves little doubt as to the correctness of the assignments of oxidation states of the latter [14]. However, it has apparently not proved possible to prepare stable, crystalline models of the [4Fe-4S] cluster in the C^{3+} oxidation level [15]. This suggests that the polypeptide chain of the high-potential protein is very effective at stabilizing the structure of the [4Fe-4S] cluster in the oxidized C^{3+} state.

Recently, X-ray crystallography and Mössbauer spectroscopy have proved a powerful combination of techniques in leading to the discovery of a novel type of iron-sulphur cluster containing three Fe atoms and three acid-labile S atoms [16,17]. A ferredoxin, called ferredoxin I, isolated from *Azotobacter vinelandii* contains a single [4Fe-4S] cluster plus the new [3Fe-3S] cluster [17]. The [4Fe-4S] cluster is reported to be EPR inactive in the protein as extracted, but develops an EPR signal on oxidation with ferricyanide at a potential of about +350 mV [18]. Therefore, this centre is assigned to a high-potential protein-like [4Fe-4S] cluster undergoing the one-electron oxidation $C^{2+} \rightarrow C^{3+}$. The [3Fe-3S] centre yields an EPR signal with an almost isotropic g factor, g 2.01, in the oxidation state of the protein as extracted [16]. On reduction with dithionite the [3Fe-3S] centre is reduced to an EPR-silent state, although magnetic Mössbauer spectra show it to be paramagnetic with an even electron spin [16].

3Fe clusters have now been identified in a number of other proteins by Mössbauer spectroscopy [20]. Especially interesting is the demonstration of a [3Fe-3S] cluster in ferredoxin, ferredoxin II, from *De-*

sulphovibrio gigas [20]. The structural details of the cluster are not known but it displays EPR and Mössbacuer spectra similar to those of the centra in *A. vinelandii* ferredoxin I. Therefore, a structure of close similarity is presumes [20].

Because the [3Fe-3S] cluster exhibits an EPR signal with a g factor just above 2.0 in the oxidized state and becomes EPR silent upon one-electron reduction, it has been confused with a [4Fe-4S] cluster of the high-potential protein type undergoing a redox cycle between the C^{3+} and C^{2+} oxidation levels [18,22]. Therefore, identification of the cluster type solely on the basis of its EPR characteristics is insecure. We have recently shown that low-temperature MCD spectroscopy is a useful method of distinguishing 3Fe and 4Fe cluster types [23,24]. MCD signals of paramagnetic species are temperature dependent, increasing in intensity as the temperature is lowered. Therefore, at low temperatures the spectrum of the paramagnetic species dominates. Furthermore, the low-temperature MCD spectra of iron-sulphur clusters are invariably more highly structured than the optical absorption spectrum and therefore provide an excellent fingerprint [23]. Analysis of the form of the magnetic field and temperature dependence yields the ground-state g factor. Hence, it is possible to identify the nature of the paramagnet being observed [25]. We have reported the low-temperature MCD spectra of oxidized and reduced high-potential protein from *C. vinosum* and the ferredoxin from *Cl. pasteurianum* [23]. The C^{3+} and C^{+} states' MCD magnetization curves can be fitted to the observed EPR g factors. Reduced high-potential protein is unambiguously diamagnetic but the low-temperature MCD spectrum of oxidized *Cl. pasteurianum* ferredoxin shows the presence of a small percentage (less than 10%) of a paramagnetic species which arises from the weak g 2.01 signal present. The low-temperature MCD spectrum of oxidized ferredoxin II from *D. gigas* is highly structured and quite distinct from that of the oxidized protein from *C. vinosum* [24]. The MCD magnetization curves fit an isotropic g factor of 2.01. The reduced form of ferredoxin II gives an intense, temperature-dependent MCD spectrum which is much less structured than that of the oxidized form. But the distinctive feature is the MCD magnetization curve which gives a steep initial slope and can be fitted to yield ground-state g factors, $g_{/\!/}$ 8.00 and $g_{\perp}$ 0.20 [24]. Hence, the MCD spectra of the two types of cluster are quite distinctive both in the form of the spectra and in their magnetization properties.

In this paper we report a study of the low-temperature MCD and EPR spectra of the ferricyanide-oxidized form of the 2[4Fe-4S] ferredoxin from *Cl. pasteurianum*, and that of the dithionite-reduced form of the ferricyanide-treated ferredoxin. It is shown that the iron-sulphur cluster, after ferricyanide oxidation, has MCD spectra in both the oxidized and reduced states identical to those of oxidized and reduced ferredoxin II from *D. gigas*. Thus, a 3Fe centre is produced from a [4Fe-4S] cluster by ferricyanide oxidation.

Materials and Methods

Materials. *Cl. pasteurianum* ferredoxin was extracted as described previously [26]. The concentration was determined from the value $\epsilon_{390} = 30\,600$ $M^{-1} \cdot cm^{-1}$ [27]. To produce the ferricyanide-oxidized state, the ferredoxin, in aqueous 0.8 M NaCl, 20 mM Tris-HCl buffer, pH 8.5, was incubated with a 3-fold molar excess of $K_3Fe(CN)_6$ at 4°C for 12 h. This treatment led to considerable loss of protein which was spun off. The solution was passed down a Sephadex G-25 column equilibrated with 20 mM Tris-HCl buffer, pH 8.5. Excess ferricyanide was removed and a bright-blue band of Prussian blue was observed at the top of the G-25 column. The protein solution was applied to a DE-23 cellulose column equilibrated with 20 mM Tris-HCl buffer, pH 8.5. The brown protein band was eluted with 0.8 M NaCl, 20 mM Tris-HCl buffer, pH 8.5.

For low-temperature MCD measurements aqueous solutions of protein in 20 mM Tris-HCl buffer, pH 8.5, were diluted to 50% (v/v) with ethanediol in order to form good optical quality low-temperature glasses. The ferricyanide-treated protein solutions were handled aerobically. Dithionite reduction was carried out anaerobically using solid sodium dithionite.

Instrumentation. Absorption spectra were recorded on a Cary 14 and a Cary 17 spectrophotometer. MCD spectra were measured with a JASCO J500D spectropolarimeter fitted with an Oxford

Instruments Ltd. SM-4 superconducting solenoid, capable of generating magnetic fields of up to 5.3 T. The magnet is a split-coil design enabling the sample to be introduced directly into a chamber of liquid helium. Further experimental details have been given elsewhere [23,25].

EPR spectra were measured with a Varian E-104, X-band spectrometer fitted with an Oxford Instruments Ltd. E.S.R.-9 flow cryostat.

MCD spectra are displayed as $\Delta\epsilon = \epsilon_L - \epsilon_R$, where ϵ_L and ϵ_R are the molar extinction coefficients for left and right circularly polarized light, respectively, or as $\Delta A = A_L - A_R$ where A is absorbance.

Results

Fig. 1 compares the EPR spectra of the following states of *Cl. pasteurianum* ferredoxin, the ferricyanide-reacted protein (Fig. 1a) and the dithionite-reduced sample of ferredoxin after ferricyanide treatment (Fig. 1b). Each sample had been diluted with ethanediol to 50% (v/v) in order to reproduce the same conditions as employed for the low-temperature MCD experiments. Freshly extracted protein shows the presence, as expected, of the weak g 2.01 signal [23]. On reduction with dithionite the intense signal typical of the 2[4Fe-4S]$^+$ ferredoxin is obtained [23]. After ferricyanide treatment the g 2.01 signal is increased by a factor of about 100 (Fig. 1a) compared with that seen in the freshly prepared ferredoxin [23]. On reduction of this sample with dithionite a very weak signal with g factors of 2.05, 1.94 and 1.89 is obtained (Fig. 1b). The integrated intensity is 21% of that of the signal in Fig. 1a. Therefore, in this preparation a small amount of a [4Fe-4S]$^+$ cluster remains. However, the signal is a simple axial one and does not show the complex features of the reduced 8Fe ferredoxin [5]. This suggests that there remains no protein with an intact complement of a pair of [4Fe-4S] clusters.

The low-temperature MCD spectra at 1.52, 2.025, 4.125 and 20 K of ferricyanide-oxidized ferredoxin are given in Fig. 2. The spectra are plotted in arbitrary units of ΔA since no ϵ values have been determined for this form of ferredoxin. The spectra are highly structured and strongly temperature dependent. From these spectra MCD magnetization curves have been constructed (Fig. 3). Curve a in Fig. 3 is

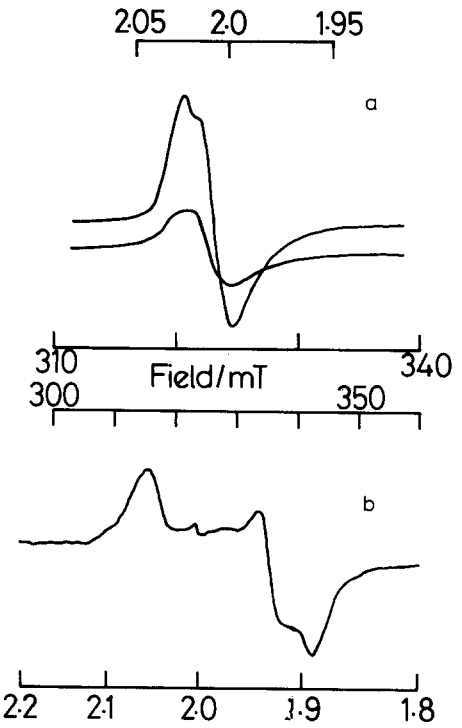

Fig. 1. (a) EPR spectrum of ferricyanide-oxidized *Cl. pasteurianum* ferredoxin recorded at 20 mW and 5 G modulation, with a gain of 15X. Upper trace recorded at 20 K, lower trace at 27 K. (b) EPR spectrum of re-reduced ferricyanide-oxidized ferredoxin recorded at 20 mW, 10 G modulation with a gain of 320X at 25 K. In each case, the sample was dissolved in 20 mM Tris-HCl, pH 8.3/0.8 M NaCl diluted to 50% with ethylene glycol. Reduction was by anaerobic addition of sodium dithionite solution and reaction for 1 min.

the intensity from the peak at 306 nm to the trough at 346 nm. Also given is the theoretically calculated magnetization curve determined for an isotropic g factor of 2.01 from a spin $S = 1/2$ ground state according to the procedures described by Thomson and Johnson [25] using the theory developed by Schatz et al. [28].

Fig. 4 shows the MCD spectra of *Cl. pasteurianum* ferredoxin which has been ferricyanide oxidized for 12 h and then anaerobically reduced with solid sodium dithionite. The spectra shown have been measured at 1.52, 1.95, 4.215, 7.5 and 17.5 K at 5.13 T. The spectra are intense and strongly temperature dependent. The intensity scale is given in arbitrary units of ΔA. From these spectra MCD magnetization

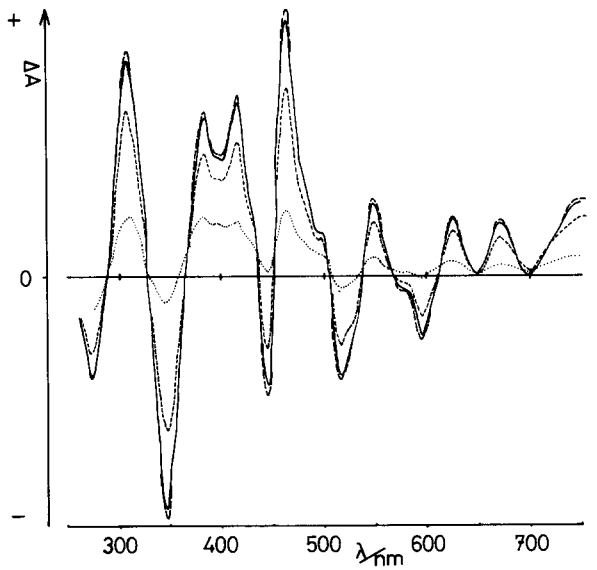

Fig. 2. MCD spectrum of ferricyanide-oxidized *Cl. pasteurianum* ferredoxin. (– – –) 1.52 K, (———) 2.025 K, (- - - - - -) 4.125 K, (· · · · · ·) 20 K. Magnetic field 5.13 T, path length 1.062 mm. The sample was dissolved in 20 mM Tris-HCl, pH 8.3/0.8 M NaCl diluted to 50% (v/v) with ethylene glycol.

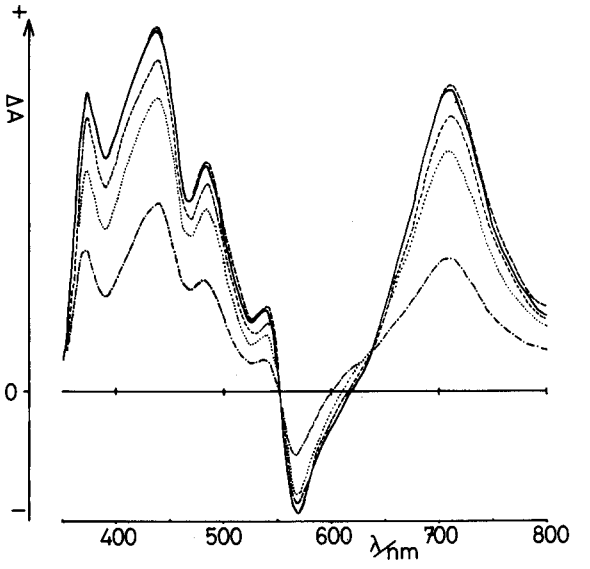

Fig. 4. MCD spectrum of re-reduced ferricyanide-oxidized *Cl. pasteurianum* ferredoxin. (– – –) 1.52 K, (———) 1.95 K, (- - - - - -) 4.215 K, (· · · · · ·) 7.5 K, (– · – · –) 17.5 K. Magnetic field 5.13 T, path length 1.264 mm. The sample was dissolved in 20 mM Tris-HCl, pH 8.3/0.8 M NaCl, made anaerobic and reduced for 5 min with solid sodium dithionite, then diluted to 50% (v/v) with ethylene glycol.

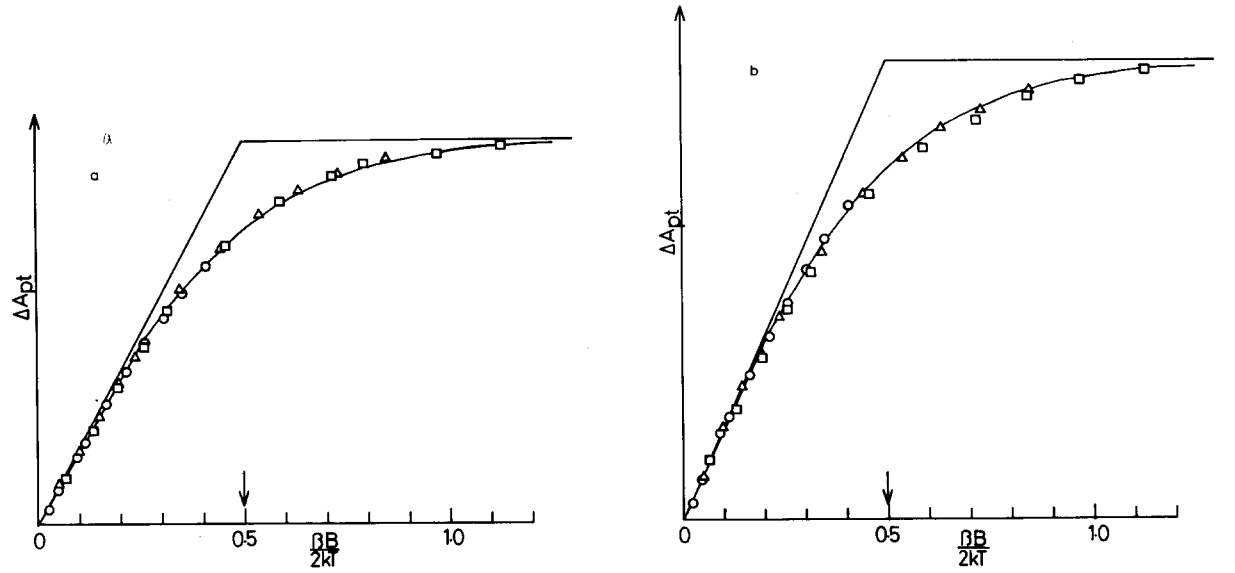

Fig. 3. MCD magnetization curves of ferricyanide-oxidized *Cl. pasteurianum* ferredoxin. ΔA_{pt} is the ΔA value measured from peak at 462 nm to trough at 444 nm (a), and from peak at 306 nm to trough at 346 nm (b) – expressed in arbitrary units. Intercept $I = 0.50$. (□) 1.52 K, (△) 2.025 K, (○) 4.215 K. The solid line is the curve computed for isotropic g 2.01 [25].

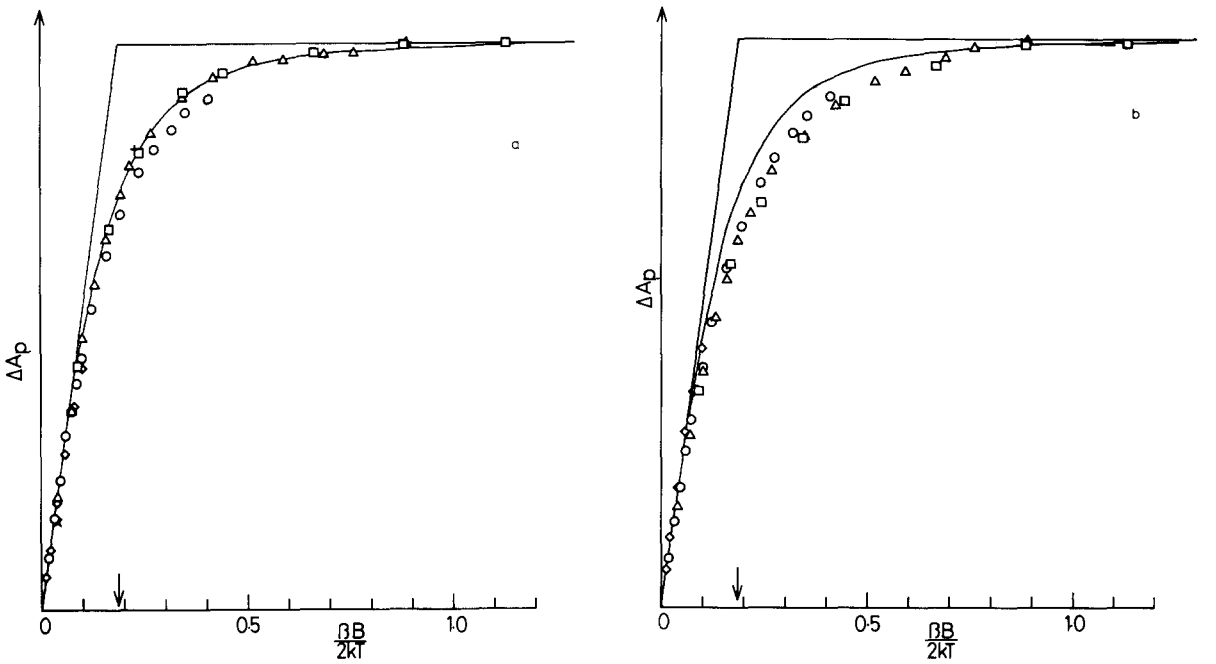

Fig. 5. MCD magnetization curves of re-reduced ferricyanide-oxidized *Cl. pasteurianum* ferredoxin. ΔA_p is the ΔA value measured at 710 nm (a) and 372 nm (b) – expressed in arbitrary units. (□) 1.52 K, (△) 1.95 K, (○) 4.215 K, (◇) 7.5 K, (X) 17.5 K, (+) 43.5 K. The solid line is the curve computed for $g_{/\!/}$ 8.0 $g_{\perp}$ 0 [24,25]. Intercept $I = 0.18$.

curves have been constructed (Fig. 5). The peaks at 710 nm (Fig. 5a) and 372 nm (Fig. 5b) have been used to plot the curves. They clearly have a much steeper initial slope and magnetize more rapidly than the curves of Fig. 3. The solid lines in both Fig. 5a and b are the curves computed for a doublet ground state with effective g factors $g_{/\!/}$ 8.0 and $g_{\perp}$ 0.0 [24,25]. The curves have been drawn to give the most satisfactory fit in the region of the initial slope and at the magnetization limit.

Discussion

As a result of the work of Sweeney et al. [12] on the EPR signals at g 2.01 of bacterial ferredoxins in the oxidised and the ferricyanide-oxidized proteins it has been generally assumed that the signals belong to a $[4Fe\text{-}4S]^{3+}$ cluster, isoelectronic with that in the oxidized high-potential protein. These workers termed this a superoxidized [4Fe-4S] cluster. The discovery of the [3Fe-3S] cluster in *A. vinelandii* ferredoxin I [16] and in ferredoxin II from *D. gigas* which also gives a EPR signal with g 2.01 in the oxidized state [20] naturally suggests an alternative interpretation of the g 2.01 signals derived from 8Fe and 4Fe ferredoxins. Therefore, a comparison of the MCD spectrum of the ferricyanide-oxidized ferredoxin of *Cl. pasteurianum* with that of oxidized ferredoxin II from *D. gigas* and with that of oxidized high-potential protein from *C. vinosum* has been made in Fig. 6. The spectra measured at 4.22 K and 5.1 T are compared. There is a striking similarity between the MCD spectra of the superoxidized *Cl. pasteurianum* ferredoxin and that of ferredoxin II from *D. gigas*. The overlap of the bands is remarkable. Although the MCD spectrum of oxidized high-potential protein is also highly structured, there is little resemblance to the other spectra. The magnetization curves (Fig. 3) confirm that the MCD spectra of the ferricyanide-oxidized form of *Cl. pasteurianum* ferredoxin arise from a paramagnetic species with an electronic ground state which is a doublet. The excellent fit to the simulated curves for an isotropic g 2.01 verifies that the EPR-detected species is dominating the MCD spectra. This leaves little doubt that the paramagnetic centres in oxidized

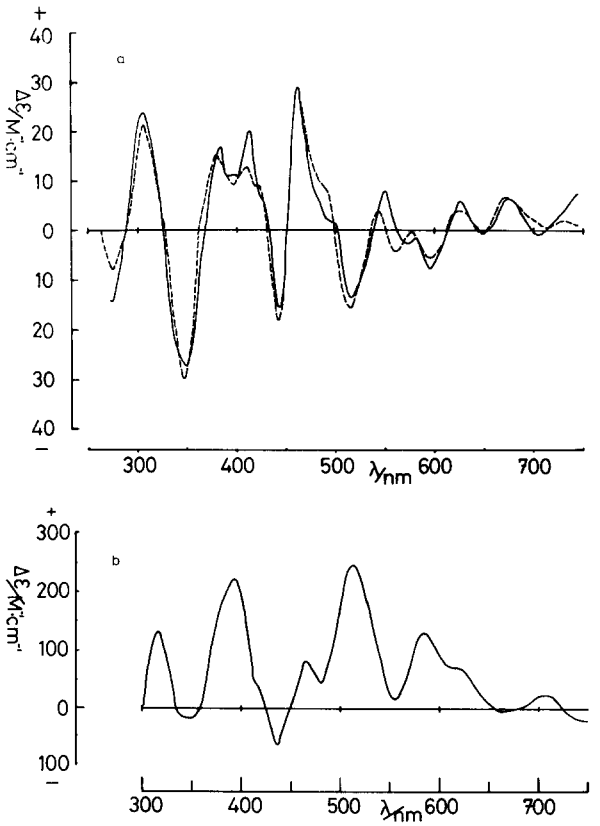

Fig. 6. (a) The MCD spectra of ferricyanide-oxidized *Cl. pasteurianum* ferredoxin (———) and oxidized ferredoxin II from *D. gigas* (- - - - -) (from Ref. 24) at 4.215 K and 5.13 K. The Δε scale refers only to the spectrum of ferredoxin II. The spectra have been normalized at 462 nm. (b) The MCD spectrum of oxidized high-potential protein from *C. vinosum* [23] at 4.215 K and 5.13 T.

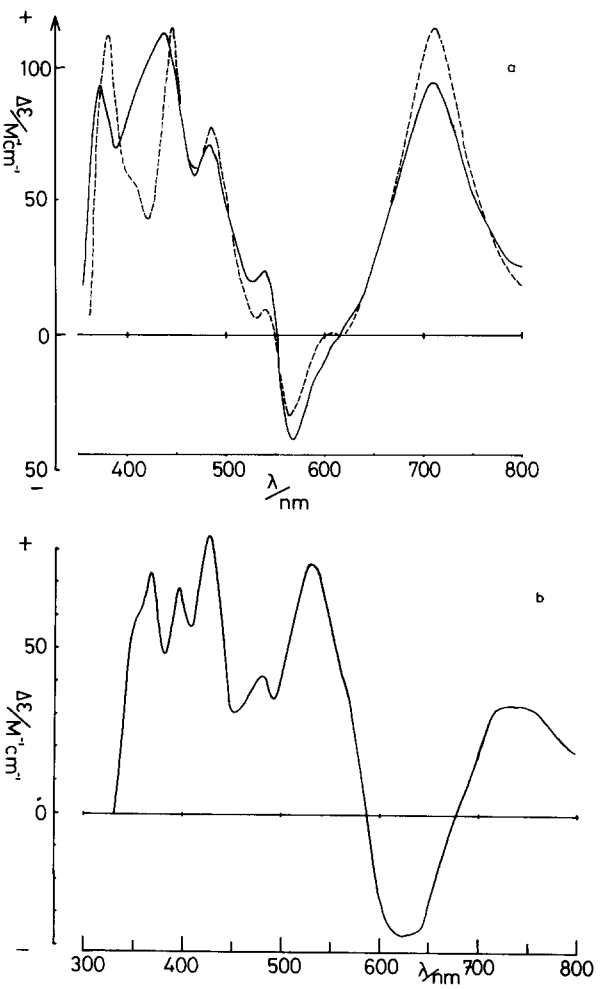

Fig. 7. (a) The MCD spectra of dithionite-reduced, ferricyanide-oxidized *Cl. pasteurianum* ferredoxin (———) and dithionite-reduced ferredoxin II from *D. gigas* (from Ref. 24) at 4.215 K and 5.13 T. The Δε scale refers only to the spectrum of ferredoxin II. The spectra have been normalized at 500 nm. (b) The MCD spectrum of dithionite-reduced *Cl. pasteurianum* ferredoxin at 4.215 K and 5.13 T (from Ref. 23).

ferredoxin II and superoxidized ferredoxin from *Cl. pasteurianum* are extremely similar if not identical. It should be noted that the MCD spectra at low temperature are dominated by the paramagnetic species. Therefore, any diamagnetic clusters present, such as $[4Fe\text{-}4S]^{2+}$, will not be detected.

Confirmation of the similarity of the iron-sulphur clusters in ferredoxin II and the ferricyanide-treated ferredoxin from *Cl. pasteurianum* is provided by the MCD spectra of these species when reduced by dithionite. Fig. 7a presents a comparison of the MCD spectra at 4.22 K and 5.1 T of reduced ferredoxin II from *D. gigas* and of dithionite-reduced, ferricyanide-treated ferredoxin from *Cl. pasteurianum*. Fig. 7b gives the MCD spectrum of dithionite-reduced ferredoxin from *Cl. pasteurianum*. The match between the spectra in Fig. 7a is not perfect. There are additional contributions to the spectrum of the ferricyanide-treated protein at 400, 500 and 700 nm. However, these are the wavelength regions in which a contribution from intact reduced [4Fe-4S] clusters will appear, as inspection of Fig. 7b reveals. The EPR spectrum of Fig. 1b shows that a small amount of intact $[4Fe\text{-}4S]^{+}$ clusters remains.

The magnetization curves of Fig. 5 confirm these conclusions. They show that the MCD spectrum is dominated by a paramagnetic species with high g factors in the ground state. An excellent fit of the curves to $g_{/\!/}$ 8.0 and $g_{\perp}$ 0.0 is obtained for the positive MCD peak at 710 nm. These are the values expected for an electronic doublet with $M_s = \pm 2$, in an axial field. Our analysis of the MCD magnetization curves of ferredoxin II from *D. gigas* [24] led to the conclusion that the reduced ferredoxin II has a ground-state spin $S = 2$, with the lowest zero-field component being the doublet $M_s = \pm 2$. In that case the best fit for the peak at 710 nm was with effective g factors $g_{/\!/}$ 8.0 and $g_{\perp}$ 0.20. Fig. 5b is the magnetization curve of the reduced form of the ferricyanide-damaged ferredoxin from *Cl. pasteurianum* measured at 372 nm. The fit of the theoretical curve for $g_{/\!/}$ 8.0 and $g_{\perp}$ 0.0 is not as satisfactory. However, this is the wavelength region in which a contribution from the $[4Fe\text{-}4S]^{+}$ cluster will be significant. Since a C^{+} cluster magnetizes as a spin $S = 1/2$ paramagnet, a contribution from such a species will tend to lower the initial slope; as is observed in Fig. 5b. Thus, the magnetization curves of Fig. 5 confirm that the MCD spectra of the dithionite-reduced form of the ferricyanide-oxidized ferredoxin of *Cl. pasteurianum* are dominated by a cluster with magnetic properties closely similar to those of reduced ferredoxin II from *D. gigas*.

These results for the two oxidation levels leave no doubt that the centre produced in ferredoxin from *Cl. pasteurianum* by ferricyanide treatment is closely similar to, if not identical with, the centre in ferredoxin II from *D. gigas* which has been established by Mössbauer spectroscopy to be a 3Fe cluster. This conclusion raises many interesting questions about the biological significance of the 3Fe cluster and also about its structural chemistry.

The ferredoxins I and II from *D. gigas* were unique in containing identically the same polypeptide chain [21]. Ferredoxin I is a trimer binding a [4Fe-4S] centre, although with an apparently variable amount of [3Fe-3S] * cluster present. Ferredoxin II is a tetrameric form which binds only the [3Fe-3S] cluster. We have now shown that the polypeptide chain of the ferredoxin from *Cl. pasteurianum* is similar in being able to bind either two [4Fe-4S] clusters or a number, as yet undetermined, of [3Fe-3S] clusters. Reconstitution of the apoproteins with iron and sulphide has been carried out by several groups [22,29,30] and always leads to the formation of [4 Fe-4 S] clusters, as Mössbauer and EPR work demonstrates. We have, as yet, no evidence concening the degree of oligomerization of the polypeptide chain in the ferricyanide-oxidized ferredoxin from *Cl. pasteurianum*. It is possible that it is the dimeric form detected by Gersonde et al. [31]. They demonstrated that this form increases in concentration as aerobic oxidative damage proceeds and that its activity in the phosphoroclastic assay is the same per mole as the native, or monomeric, ferredoxin. The possibility must therefore be considered that ferredoxin II from *D. gigas* arises as a result of oxidative damage to ferredoxin I during extraction as has been discussed previously [22]. However, it has been shown that ferredoxin II is more active than ferredoxin I in the activation of sulphite reduction by sulphite reductase and hydrogenase [21].

The weak g 2.01 signal invariably present in the EPR spectra of freshly extracted bacterial ferredoxins arises from oxidative damage to the protein [12]. This centre is undoubtedly similar to the species produced by ferricyanide oxidation but several pieces of evidence suggest it is not identical. For example, Sweeney et al. [12] report that this signal power saturates much more readily than the signal from the reduced ferredoxin of *Cl. acidi-urici*, although the g 2.01 signal produced by ferricyanide treatment power saturates much less easily. Similarly, the MCD spectrum of the paramagnetic component in freshly extracted *Cl. pasteurianum* ferredoxin is similar to but not identical with the spectrum of the ferricyanide-oxidized species [23]. However, we have consistently found that the MCD spectra of the reduced ferredoxin from *Cl. pasteurianum* and *B. stearothermophilus* (a 4Fe centre) yield MCD magnetization curves which cannot be fitted to an $S = 1/2$ spin system with g factors close to 2.0. The initial slopes of the curves are invariably too steep as would be the case if the samples were contaminated by a [3Fe-3S] cluster. Although more work is required, the evidence points to a 3Fe centre of similar structure to that of ferredoxin II from *D. gigas* being

* The 3Fe cluster in ferredoxin II from *D. gigas* is written in the shorthand notation [3Fe-3S]. However, the exact number of S^{2-} in the cluster has not yet been established.

produced in 8Fe and 4Fe ferredoxins on oxidative damage. The species arising from such damage have been studied with Mössbauer spectroscopy by Gersonde et al. [30] but they concluded that the iron was not involved in cluster formation.

The structure of the 3Fe centre in *A. vinelandii* ferredoxin I, originally reported by Stout and coworkers [17], has now been refined by them [19] and shown to consist of a triangular arrangement of three Fe atoms bridged by three S^{2-} with six ligands, namely, five cysteine anions and possibly one glutamic acid carboxyl group, binding the cluster core to the protein chain. It is suggested that an important feature in the sequence which stabilizes a 3Fe core is Cys-X-Glu-X-Cys-Pro. This provides three of the six ligands. This sequence is also found in the *D. gigas* ferredoxin II polypeptide chain [32] and in at least four other ferredoxins now suspected to contain a 3Fe centre [19]. However, such a sequence is not present in the polypeptide chain of the ferredoxin from *Cl. pasteurianum*. There is a sequence $\overset{14}{\text{Cys-X-X-Glu-Cys-Pro}}\overset{19}{}$ [33] and, interestingly, the glutamic acid residue is invariant in the sequences of seven anaerobic bacteria with the sole exception of that from *Megasphaera elsdenii* [34]. The proline residue is invariant in all seven ferredoxins. Possibly, this sequence can fulfill the same role as that in ferredoxin II. However, it should be borne in mind that the ligands binding the [3Fe-3S] cluster core in ferredoxin II have not been positively identified and it is doubtful whether the Mössbauer spectrum could distinguish between a set of ligands comprising five cysteine residues and one carboxyl group and, say, six cysteine residues. The MCD spectrum of the oxidized ferredoxin could well be sensitive to such a change and we await with interest the results of the measurement of the low-temperature MCD spectra of ferredoxin I from *A. vinelandii*, which contains the crystallographically defined 3Fe centre.

Finally, we emphasize one obvious conclusion from this work. Ferricyanide can be a damaging oxidant to employ in iron-sulphur chemistry. We suspect that it is especially efficient in converting a [4Fe-4S] cluster to a 3Fe cluster because of a combination of its oxidizing power, to generate transiently an unstable C^{3+} cluster, and its ability to pull an Fe^{2+} out of a cluster in this oxidation level. This leaves a 3Fe cluster with all iron atoms in the ferric state and Prussian blue is precipitated. There is clearly a need to avoid the use of ferricyanide and to find alternative oxidizing agents which are not damaging in this way. However, it may be that the ability of $Fe(CN)_6^{3-}$ to carry out this reaction could become a useful tool in the study of the structural chemistry of the clusters. These studies suggest that some of the published work in which ferricyanide has been used as an oxidant may require reinterpretation. The important studies of the ferredoxin I from *A. vinelandii* are a case in point [18].

In the case of high redox potential protein from *C. vinosum*, the protein appears to be able to withstand damage from ferricyanide oxidation. However, there is evidence that if the protein is treated with dimethyl sulphoxide to denature it then a g 2.01 signal is formed (Cammack, R. unpublished data). It could be that, as work on inorganic models suggests, the C^{3+} state [4Fe-4S] cluster is intrinsically unstable without the environment of the appropriate protein chain.

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